PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/012 — A1

(11) International Publication Number: WO 96/40234

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/IB95/00446

(22) International Filing Date: 7 June 1995 (07.06.95)

(71) Applicant (*for all designated States except US*): PFIZER INC. [US/US]; 235 East 42nd Street, New York, NY 10017 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): EVANS, Nigel, A. [US/US]; 34 Willow Lane, East Lyme, CT 06333 (US). FINDLY, R., Craig [US/US]; 14 Warner Place, Wethersfield, CT 06109 (US). WEBER, Frederick, H. [US/US]; 159 Phoenix Court, Terre Haute, IN 47803 (US).

(74) Agents: SPIEGEL, Allen, J. et al.; Pfizer Inc., Patent Dept., 235 East 42nd Street, New York, NY 10017 (US).

(81) Designated States: CA, FI, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: IN OVO VACCINATION AGAINST COCCIDIOSIS

(57) Abstract

The invention relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo* an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof. In a preferred embodiment, the domesticated bird that is vaccinated is a chicken or turkey.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

IN OVO VACCINATION AGAINST COCCIDIOSIS

Background of the Invention

The present invention relates to a method of vaccinating domesticated birds against coccidiosis. In particular, the invention relates to the *in ovo* administration of live *Eimeria* spp sporozoites or merozoites, or mixtures thereof, into the developing eggs of domesticated birds in order to immunize the hatched chicks against coccidiosis.

Coccidiosis is an enteric disease of domesticated birds caused by infection with intracellular protozoan parasites of the genus *Eimeria*. Coccidiosis is the most economically devastating parasitic disease of domesticated birds. It is estimated that anticoccidial medications and losses due to coccidiosis cost the poultry industry hundreds of millions of dollars every year.

Various attempts to vaccinate domesticated birds against coccidiosis have been reported since the early 1950's. Current vaccination methods include administering live *Eimeria* oocysts to birds through feed or water. These methods, however, are inconvenient and inefficient because not all birds get the intended oocyst dose and many are either unprotected by the vaccine or receive a pathogenic infection.

In J. M. Sharma and B. R. Burmester, Avian Dis. 26: 134-149, 1981, the authors reported that chickens vaccinated *in ovo* with herpesvirus of turkey developed immunity against subsequent challenge with Marek's disease virus. In European patent publication no. 291173, an immunization process is referred to wherein a nonreplicating immunogen is administered *in ovo*. The immunogens specifically referred to in the European patent are a genetically engineered *Eimeria* antigen and an *Eimeria* oocyst extract. The European patent specifically excludes live parasite stages such as those used in the vaccination method claimed herein.

The present vaccination method involves *in ovo* administration of live *Eimeria* sporozoites or merozoites, or a mixture thereof, into the developing eggs of domesticated birds. The available literature suggests that such a vaccination method would be ineffective *in ovo* and should be applied post-hatch. In T.K. Jeffers and G.E. Wagenbach, J. Parasit. 56(4): 656-662, 1970, the authors reported that *in ovo* injection of *E. tenella* sporozoites on day 10 of incubation provided no significant immunological protection against subsequent challenge with *E. tenella* oocysts. In

fact, they reported that chicks that received no treatment had a greater survival rate against subsequent challenge with *E. tenella* oocysts than chicks that had been treated *in ovo* with sporozoites. In K.L. Watkins et al., Proc. VI th. International Coccidiosis Conf., Abstract E1-2, Ontario, Canada, 1993, the authors described *in ovo* inoculation with live *E. maxima* sporocysts and sporulated oocysts and concluded that their study provided no evidence that *in ovo* exposure protects against subsequent coccidial challenge with *E. maxima* oocysts 10 days post-hatch. Watkins et al. further concluded that significant immunological protection is provided if inoculation is done soon after hatch rather than *in ovo*. Contrary to this teaching, the *in ovo* vaccination method of the present invention provides unexpected immunity that protects the hatched birds against subsequent coccidial challenge.

Summary of the Invention

The present invention, also referred to herein as the "present vaccination method", relates to a method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof.

The term "domesticated bird(s)", as used herein, unless otherwise indicated, includes chickens, turkeys, ducks, game birds (including, but not limited to, quail, pheasants, and geese) and ratites (including, but not limited to, ostrich).

The term "*in ovo*", as used herein, unless otherwise indicated, means into a domesticated bird egg containing a live, developing embryo.

The term "administering *in ovo*" or "*in ovo* administration", as used herein, unless otherwise indicated, means administering the vaccine described herein to a domesticated bird egg containing a live, developing embryo by any means of penetrating the shell of the egg and introducing the vaccine. Such means of administration include, but are not limited to, injection of the vaccine.

The term "final quarter of incubation", as used herein, unless otherwise indicated, means the final quarter of incubation of a developing egg of a domesticated bird.

The term "*Eimeria*", as used herein, unless otherwise indicated, means one or more species of the genus *Eimeria* that infect domesticated birds. Such *Eimeria* species include those that are found in chicken, including *E. tenella*, *E. acervulina*,

E. maxima, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*, and also those that are found in turkeys, including *E. meleagrimitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*, and also *Eimeria* species that infect other domesticated birds as defined above. The term "*Eimeria*" also includes all strains of the foregoing species of *Eimeria*, including, but not limited to, precocious strains, and attenuated strains, which includes strains that have been irradiated, or otherwise treated, so that they fail to complete development. The term *Eimeria* also includes any newly-discovered strains or species of *Eimeria* that infect domesticated birds as defined above.

The terms "sporozoites", "sporocysts", "oocysts", and "merozoites", as used herein, unless otherwise indicated, mean live *Eimeria* sporozoites, sporocysts, oocysts, and merozoites.

The term "effective immunizing dose", as used herein, unless otherwise indicated, means a number of sporozoites or merozoites, or, when mixed, a number of sporozoites and merozoites, sufficient to provide immunological protection in the hatched birds that is greater than the inherent immunity of non-immunized birds. As used herein, the terms "immunize" and "vaccinate" are synonymous and are used interchangeably.

A preferred dose to be administered in accord with the method of the invention comprises 10 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to 10^6.

A more preferred dose comprises 10^3 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^3 to 10^6.

Another preferred dose comprises 10^2 to 10^5 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^2 to 10^5.

A preferred domesticated bird to be vaccinated in accord with the method of the invention is a chicken.

A preferred dose to be administered *in ovo* to chicken eggs comprises sporozoites r merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*. Th dose preferably includes from 10

to 10^6 sporozoites or merozoites, or a mixture thereof, for each species that is included in the dose.

Another preferred domesticated bird to be vaccinated in accord with the method of the invention is a turkey.

A preferred dose to be administered *in ovo* to turkey eggs comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. meleagrimitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*. The dose preferably includes from 10 to 10^6 sporozoites or merozoites, or a mixture thereof, for each species that is included in the dose.

Other preferred domesticated birds to be vaccinated in accord with the method of the invention are game birds, ducks and ratites.

The method of the invention further comprises, in combination with the present vaccination method, administering *in ovo* an immune stimulant at any time during incubation.

A preferred method of administering the immune stimulant is simultaneously with the *in ovo* administration of a dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites, during the final quarter of incubation.

Detailed Description of the Invention

The present vaccination method involves the *in ovo* administration, during the final quarter of incubation, of live *Eimeria* sporozoites or merozoites, or a mixture thereof, into domesticated birds' eggs. In the case of chickens, *in ovo* administration is preferably done on days 15-20 of incubation, and most preferably on day 18 of incubation. In the case of turkeys, *in ovo* administration is preferably done on days 21-26 of incubation.

The present vaccination method can be performed using any suitable *in ovo* administration method. Preferably, the present vaccine is administered via injection. According to one method of injection, a hole is made in the egg shell at the large end of the egg using an 18 guage needle to expose the egg's air cell. A 1.0-1.5 inch 22 guage needle attached to a syringe of appropriate size (1-3 ml) can be inserted through the hole and through the membrane of the air cell. An appropriate number of sporozoites or merozoites, or, when mixed, an appropriate number of sporozoites

and merozoites, are suspended in a suitable liquid carrier, for instance 10-500μl f phosphate-buffered saline, and then injected into the egg. The appropriate volume will depend on the size of the egg being treated, with ostrich eggs obviously being capable of taking more volume than chicken eggs. The site of injection can be within any region of the egg or embryo. Preferably, injection is done axially through the center of the large end of the egg into the amnion.

Alternatively, an automated egg injection system can be used in the present vaccination method. Such systems are described in U.S. Patent nos. 4,681,063, 4,040,388, 4,469,047, and 4,593,646, which are herein incorporated by reference. Other appropriate methods of injection are known to those skilled in the art.

Oocysts can be prepared by any of several methods known to those skilled in the art. Such methods include those described in J.F. Ryley et al., Parasitology 73:311-326, 1976 and P.L. Long et al., Folia Veterinaria Latina VI#3, 201-217, 1976, which are herein incorporated by reference. According to one method, commercial broiler chickens, approximately 2 weeks old, are infected with the *Eimera* species of interest by oral gavage of an appropriate dose of sporulated oocysts. For example, a typical dose used for *E. tenella* is 200,000 sporulated oocysts/bird. Well known procedures for collection and purification of oocysts from infected birds are then followed. For most species of *Eimeria*, feces are collected from infected birds 5-7 days post-infection, blended and filtered to remove debris, then centrifuged at a speed sufficient to pellet the remaining fecal material. For *E. tenella*, a similar procedure is used except that cecal cores are taken at 6 days post-infection. The pellet is resuspended in a saturated salt solution, in which the oocysts float and most of the contaminating debris can be removed by centrifugation. The oocyst suspension is then diluted to lower the salt concentration. The oocysts are washed repeatedly to remove the salt and resuspended in potassium dichromate solution (2.5% w/v). The oocyst suspension is incubated at 29°C with shaking (e.g., 140 rpm) for approximately 72 hours to induce sporulation of the oocysts. Alternatively, the oocysts can be treated with sodium hypochlorite and then sporulated. The number of sporulated oocysts/ml is determined by direct count using a hemocytometer, and the culture is stored, preferably under refrigeration until needed.

To prepare sporocysts, the potassium dichromate is removed from the oocyst suspension described above by repeated washing of the oocysts, which involves collection of oocysts by centrifugation and resuspending in deionized or distilled water. When the dichromate has been removed as judged by the lack of yellowish-orange coloration, the oocyst suspension is mixed with an equal volume of sodium hypochlorite (bleach) and incubated at room temperature for 15 minutes. The bleach is then removed by repeated washings, and the oocysts are resuspended in physiological saline or deionized water. Oocysts can be broken to release sporocysts using a variety of known techniques. For example, oocysts can be broken to release sporocysts by mixing the oocysts with glass beads of 1-4 mm diameter and shaking by hand, vortex mixer, or shaking incubator, or using a hand-held homogenizer. Unbroken oocysts and oocysts walls can be separated from the released sporocysts by differential centrifugation in 50% Percoll™ (sold by Pharmacia Biotech) or 1 M sucrose as described in Dulski et al., Avian Diseases, 32: 235-239, 1988.

To prepare sporozoites or a sporozoite-rich preparation to be used in accord with the present invention sporozoites are excysted from the sporocyst preparation described above. In one procedure, sporocysts prepared as described above are pelleted by centrifugation, resuspended in excystation buffer (0.5% taurodeoxycholic acid and 0.25% trypsin in phosphate buffered saline, pH 8.0) and incubated with shaking for one hour at 41°C. A sample of the resulting suspension is counted to determine the number of sporozoites, the sporozoites are washed once to remove the excystation buffer, and resuspended in phosphate-buffered saline at the desired concentration for *in ovo* injection. This preparation contains sporozoites, sporocysts and oocysts, and, without further purification, can be used in accord with the present vaccination method. Purified sporozoites, removed from sporocysts and oocysts, can be prepared by DE-52 anion exchange chromatography as described in D.M. Schmatz et al., J. Protozool. 31: 181-183, 1984, which is herein incorporated by reference. The dose of sporozoites to be used in the present vaccination method will vary according to the type of domesticated bird being vaccinated and the species of *Eimeria* being used in the vaccine. In general, the dose can range from 10 to 10^6 sporozoites per egg. Preferably, the dose ranges from 10 to 10^5 sporozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 sporozoites per egg.

Merozoites can be prepared by various methods known to those skilled in the art. In one method, sporozoites are infected into primary chick kidney cells (PCK) that are grown in culture as cell aggregates, using a modification of the method described in D. J. Doran, J. Parasit. 57: 891-900, 1971, which is herein incorporated by reference. PCK cells are grown at 40°C in 3% CO_2 in modified RK2 medium - DMEM/F12 with L-glutamine and 15 mM HEPES, supplemented with fetal bovine serum, penicillin-streptomycin, 15 mM sodium bicarbonate, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenious acid and 0.01 μM hydrocortisone HCL as described in S.D. Chung et al., J. Cell Biol. 95: 118-126, 1982. PCK cells are prepared from kidneys of 2 to 3 week old chicks by mincing the kidney, and then treating the tissue at 37°C with several changes of 0.2 mg/ml collagenase (sold by Worthington Biochemical Corp., Freehold, NJ) in phosphate-buffered saline solution. The cellular aggregates in the supernatant are washed, resuspended in modified RK2 medium containing 5% fetal bovine serum and used to seed tissue cultures flasks at a density of 10^5 aggregates per cm^2. The PCK cells are incubated for 18 hours at 40°C in 3% CO_2 and then infected with 4×10^5 sporozoites/cm^2. Infected cultures are grown in modified RK2 medium containing 2% fetal bovine serum. After 24 hours of incubation, to allow invasion, uninvaded sporozoites are removed by agitating the flask and removing the culture medium. The cell layer is washed once with modified RK2 medium containing 2% fetal bovine serum and the culture medium is again discarded. Fresh RK2 medium is added, and the cultures are incubated for another 48 to 54 hours until merozoites are released into the culture medium.

Purification of the merozoites to remove host cell debris can be done by various methods known to those skilled in the art. In accord with one method, as described in J.A. Olson, Antimicrob. Agents Chemother. 34: 1435-1439, 1990, the culture medium containing the released merozoites is collected and spun at 450 X g for 10 minutes to concentrate the merozoites. The pellet containing the merozoites and the host cell debris is suspended in 0.1 M NaCl-0.05 M KCl-20% bovine serum albumin and applied to a DE-52 anion exchange column equilibrated in 75 mM Tris-40 mM NaH_2PO_4-86 mM NaCl-100 mM glucose at pH 8.2. Merozoites flow through the column. Merozoites collected from the column can be tested for purity by electron microscopy as described in A. Kilejian, J. Biol. Chem. 249: 4650-

4655, 1974. In general, the merozoite dose can range from 10 to 10^6 merozoites per egg. Preferably, the dose ranges from 10 to 10^5 merozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 merozoites per egg. When sporozoites and merozoites are mixed, in general, the dose comprising the total number of merozoites and sporozoites can range from 10 to 10^6 per egg. Preferably, the dose ranges from 10 to 10^5 merozoites and sporozoites per egg, and, more preferably, the dose ranges from 10^2 to 10^5 merozoites and sporozoites per egg.

The sporozoites or merozoites, or mixture thereof, can be injected *in ovo* in any physiologically suitable medium. Preferably, they are suspended in physiologically balanced saline such as phosphate-buffered saline. The selected medium can optionally include one or more suspending agents including physiologically suitable gels, gelatins, hydrosols, cellulose, or polysaccharide gums.

Preferably, in the present vaccination method, sporozoites or merozoites, or a mixture thereof, of two or more *Eimeria* species are injected *in ovo* at the same time. In accord with the present vaccination method, sporozoites or merozoites, or a mixture thereof, of all identified species of *Eimeria* that infect a specific domesticated bird, such as chicken, can be injected *in ovo* at the same time, or in series, at appropriate doses to provide immunological protection against all species.

Immune stimulants can be used in conjunction with the present vaccination method. Suitable immune stimulants include, but are not limited to, cytokines, growth factors, chemokines, supernatants from cell cultures of lymphocytes, monocytes, or cells from lymphoid organs, cell preparations or cell extracts (e.g. *Staphylococcus aureus* or lipopolysaccharide preparations), mitogens, or adjuvants including low molecular weight pharmaceuticals. An immune stimulant can be administered *in ovo* at any time during incubation. Preferably, an immune stimulant is administered *in ovo* in the medium containing the dose of *Eimeria* sporozoites or merozoites, or mixture thereof.

The efficacy of the present invention in vaccinating against coccidiosis is illustrated in the following examples. Each dose was injected *in ovo* in physiologically-acceptable saline as described above. The effectiveness of a particular preparation was determined by monitoring its effect on hatch rate and hatch weight of the chicks, and, following challenge infection, oocyst production, weight gain, and pathogenicity (lesion score). Lesion scores were assigned

according to the protocol described in J.K. Johnson and W.M. Reid, Exp. Parasitol. 28: 30-36, 1970, according to which a value of 0 represents no disease and a value of 4 represents maximum pathology.

EXAMPLE 1

Chicken eggs were injected on day 18 of incubation with a preparation containing 10^5 *E. tenella* sporozoites per egg. The preparation was not purified to remove sporocysts and oocysts. Each dose also contained approximately 10^4 *E. tenella* sporocysts and approximately 10^4 *E. tenella* oocysts. As a control, eggs were injected only with phosphate-buffered saline solution. In the sporozoite-treated population of birds the mean oocyst shed at 7 days post-hatch was 1.1×10^6 oocysts/bird. Non-immunized birds and the sporozoite-treated birds were challenged with various doses of sporulated oocysts of *E. tenella*, administered by oral gavage, on days 7, 14 or 21 post-hatch. The data appear in Table 1.

Immunized Versus Non-Immunized Birds: Responses To Different Challenge Doses At Different Times Post-Hatch

TABLE 1

Group[1]	Challenge dose (sporulated oocysts per bird)	Lesion score on sixth day after challenge		Weight gain (grams) per bird over six day period after challenge	
		Non-immunized	Immunized *In Ovo* (10^5 sporozoites on day 18 of incubation)	Non-immunized	Immunized *In Ovo* (10^5 sporozoites on day 18 of incubation)
Challenge on day 7 post-hatch	0	0	0	188	167
	2.5×10^3	3.0	0.3*	154	153
	5×10^3	3.1	1.2*	153	167
	1×10^4	3.7	1.4*	123	165*
Challenge on day 14 post-hatch	0	0	0	266	278
	2.5×10^3	2.4	0.4*	260	269
	5×10^3	2.8	1.1*	244	265
	1×10^4	3.3	1.6*	235	259
Challenge on day 21 post-hatch	0	0.1	0	361	357
	2.5×10^3	2.3	0.7*	375	358
	5×10^3	2.1	0.8*	332	344
	1×10^4	2.8	1.4*	337	395*

[1] Each group was subjected to a single challenge of sporulated oocysts on days 7, 14, or 21 post-hatch. For example, birds challenged on day 21 post-hatch were not challenged on days 7 or 14 post-hatch.

* Significantly different from non-immunized birds ($p<0.05$, ANOVA)

The data in Table 1 clearly show that the immunized birds were less susceptible to infection than their non-immunized hatch mates as indicated by the reduced lesion scores and improved weight gains in the immunized birds. The data also demonstrate that the method of the invention imparts immunity to the chicks at a relatively early age (within seven days post-hatch). Furthermore, the data show that the immunity continues as the chicks grow and develop. Imparting immunity to chicks at an early age provides a significant advantage in the broiler chicken industry because broilers routinely reach market by 6 weeks of age.

EXAMPLE 2

Chicken eggs were injected on day 18 of incubation with saline (control) or preparations containing different doses of sporozoites of *E. tenella*, as indicated in Table 2 which provides the pre-challenge results. The sporozoite preparation used for each dose contained 62% sporozoites, 9% sporocysts, and 29% oocysts. Each dose containing 10^5 sporozoites included a total of 1.6 X 10^5 parasite stages (sporozoites, sporocysts, and oocysts).

Effect Of *In Ovo* Vaccination On Hatch Rate And Weight

TABLE 2

Sporozoites injected per egg on day 18 of incubation	Hatch rate (%)	Hatch weight (grams)
0	94	48.2
0	94	48.0
10^3	100	49.4
10^4	97	47.0
10^5	94	49.1

The data in Table 2 show that chicks hatched from eggs injected with live sporozoites were substantially identical to their non-immunized hatch mates in terms of hatch rate and hatch weight. The chicks were then challenged on day 14 post-

hatch with 1.25 X 10^4 sporulated oocysts of *E. tenella* per bird, administered by oral gavage. The post-challenge results are provided in Table 3.

Response To Challenge Infection Of Non-Immunized Birds Versus Birds Treated *In Ovo* With Different Doses Of Sporozoites

TABLE 3

Sporozoites injected per egg on day 18 of incubation	Weight Gain per bird over six day period after challenge	Lesion Score on day 6 after challenge	Oocysts shed per bird (x 10^6) on day 6 after challenge
0	278	3.2	12.3
0 (Unchalleng -ed control)	321	0	0.003*
10^3	289	2.6*	11.2
10^4	291	2.7	12.2
10^5	304*	1.4*	1.4*

* Significantly different from non-immunized group (received saline) that was subjected to challenge ($p<0.05$, ANOVA)

The data in Table 3 show that for every parameter (weight gain, lesion score, oocyst shed) chicks hatched from eggs injected with different doses of the sporozoite preparation showed evidence of immunity. In comparison to the control birds that were treated only with saline and were subjected to challenge infection, the birds immunized *in ovo* with the sporozoite preparation showed greater weight gain and reduced lesion scores. In addition, the birds immunized *in ovo* with the sporozoite preparation passed fewer oocysts than the control birds after challenge infection, indicating that the infection was less severe in the immunized birds.

We claim:

1. A method of vaccinating a domesticated bird against coccidiosis comprising administering *in ovo*, during the final quarter of incubation, an effective immunizing dose of live *Eimeria* sporozoites or merozoites, or a mixture thereof.

2. The method of claim 1 wherein the dose comprises 10 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10 to 10^6.

3. The method of claim 1 wherein the dose comprises 10^3 to 10^6 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^3 to 10^6.

4. The method of claim 1 wherein the dose comprises 10^2 to 10^5 sporozoites or merozoites, or a mixture thereof wherein the total number of said sporozoites and merozoites ranges from 10^2 to 10^5.

5. The method of claim 2 wherein the domesticated bird is a chicken.

6. The method of claim 5 wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species of *Eimeria* selected from the group consisting of *E. tenella*, *E. acervulina*, *E. maxima*, *E. necatrix*, *E. mitis*, *E. praecox*, and *E. brunetti*.

7. The method of claim 6 wherein the dose is administered by *in ovo* injection.

8. The method of claim 2 and further comprising administering *in ovo* an immune stimulant at any time during incubation.

9. The method of claim 8 wherein the immune stimulant is administered *in ovo* simultaneously with the dose of sporozoites or merozoites, or mixture of said sporozoites and merozoites.

10. The method of claim 2 wherein the dose comprises merozoites.

11. The method of claim 2 wherein the dose comprises sporozoites.

12. The method of claim 11 wherein the sporozoites have been purified to remove sporocysts and oocysts.

13. The method of claim 2 wherein the domesticated bird is a turkey.

14. The method of claim 13 wherein the dose comprises sporozoites or merozoites, or a mixture thereof, of two or more species f *Eimeria* selected from the

group consisting of *E. meleagrimitis*, *E. adenoeides*, *E. gallopavonis*, *E. dispersa*, *E. meleagridis*, *E. innocua*, and *E. subrotunda*.

15. The method of claim 14 wherein the dose is administered by *in ovo* injection.

16. The method of claim 2 wherein the domesticated bird is a game bird, duck or ratite.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IB 95/00446

A. CLASSIFICATION F SUBJECT MATTER
IPC 6 A61K39/012

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 291 173 (EMBREX INC.) 17 November 1988 cited in the application see page 2 - page 3	1-16
A	THE JOURNAL OF PARASITOLOGY, vol. 56, no. 4, 1970 pages 656-662, T.K.JEFFERS ET AL. 'Embryonic Response to Eimeria Tenella Infection' cited in the application see abstract	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1995	12.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Tzschoppe, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ..onal Application No
PCT/IB 95/00446

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LES COLLOQUES DE L'INRA, vol. 49, 1989 pages 655-660, T.L.FREDERICKSEN ET AL. 'In Ovo administration of a potent recombinant coccidial antigen vaccine in poultry' see abstract ---	1-16
A	EP,A,0 650 733 (AMERICAN CYANAMID COMPANY) 3 May 1995 see page 2 - page 7 ---	1-16
A	WO,A,94 16725 (MERCK & CO, INC.) 4 August 1994 see page 1 - page 2 -----	1-16

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 95/00446

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-291173	17-11-88	CA-A-	1326818	08-02-94
		JP-A-	1025731	27-01-89
		ZA-A-	8802708	17-10-88
EP-A-650733	03-05-95	AU-B-	7763294	18-05-95
		CA-A-	2134835	04-05-95
		JP-A-	7206705	08-08-95
WO-A-9416725	04-08-94	AU-B-	5989894	15-08-94